Int. J. Electrochem. Sci., 5 **(2010)** 1422 - 1433

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

FFT Continuous Cyclic Voltammetry Triglyceride Dual Enzyme Biosensor Based on MWCNTs-CeO_2 Nanoparticles

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Received: 16 August 2010 / *Accepted:* 19 August 2010 / *Published:* 1 October 2010

A new electrochemical biosensor designed for the triglyceride determination based on nanomaterials. Lipase and glycerol dehydrogenase enzymes were used as sensing materials and they were immobilized on to the CeO_2 nanoparticles and Multiwall cabon-nanotubes (MWCNTs) placed on a glassy carbon electrode by nafion. The detection method was based on fast Fourier transform (FFT) continuous cyclic voltammetry in a flow injection system. Under optimal detection conditions, the linear response was in the range of 1 to 100 mg/L with detection limit of 0.5 mg/L at a signal-to-noise ratio of 3. The biosensor showed an acceptable reproducibility and good stability.

Keywords: Triglyceride, biosensor, nanoparticles, FFT Cyclic voltammetry

1. INTRODUCTION

Recently, fast Fourier transform method was found very sensitive system in combination by electrochemical method for trace detection of compounds [1-5]. Most of the electrochemical (EC) signals are continuous, that is, they have a defined value for every possible instant in time. Analog voltage and current are two examples of continuous-time signals that are measured by using instrumentation. However, in order to analyze these continuous signals using a computer-based measurement system, the signal should convert into digital signal, with each sample representing a numeric value that is proportional to the measured signal at a specific instant in time which is converting the continuous-time signal into a discrete-time signal. The sampling process employed for electrochemical measurements creates digital signal data spaced on an even interval of time. Fourier

Transform (FT) is a defined technique for converting or transforming EC data from the time domain into the frequency domain.

In particular, when the magnitude of current is in the range of nano and pico ampere, electrochemical response suffers from existence of environmental noises. The approach used here is designed to separate the voltammetric signal and background signal in frequency domain by using discrete Fast Fourier Transformation (FFT) method. Based on FFT information, the cutoff frequency of the analog filter is set at a certain value (where the noises are removed from EC data). This separation allows, digitally filtrating some of the noises and decreasing the bandwidth of the measurement. In new EC methods, the potential waveform was continuously applied where the collected data are filtered by the FFT, before their use in the signal calculation. For calculation of the signal of the analyte and noise reduction, a special computer based numerical method is also introduced. The calculation of signal was based on the net partial and total charge exchanges at electrode surface and was done by integrating the currents at the selected potential range at the voltammogram [6-9].

This work introduces a new electrochemical biosensor for determination of triglyceride combine with FFT continuous cyclic voltammetry (FFTCCV) technique in a flow injection analysis system. To the best of our knowledge, this is the first application of FFTCCV method as detection technique of a triglyceride biosensor.

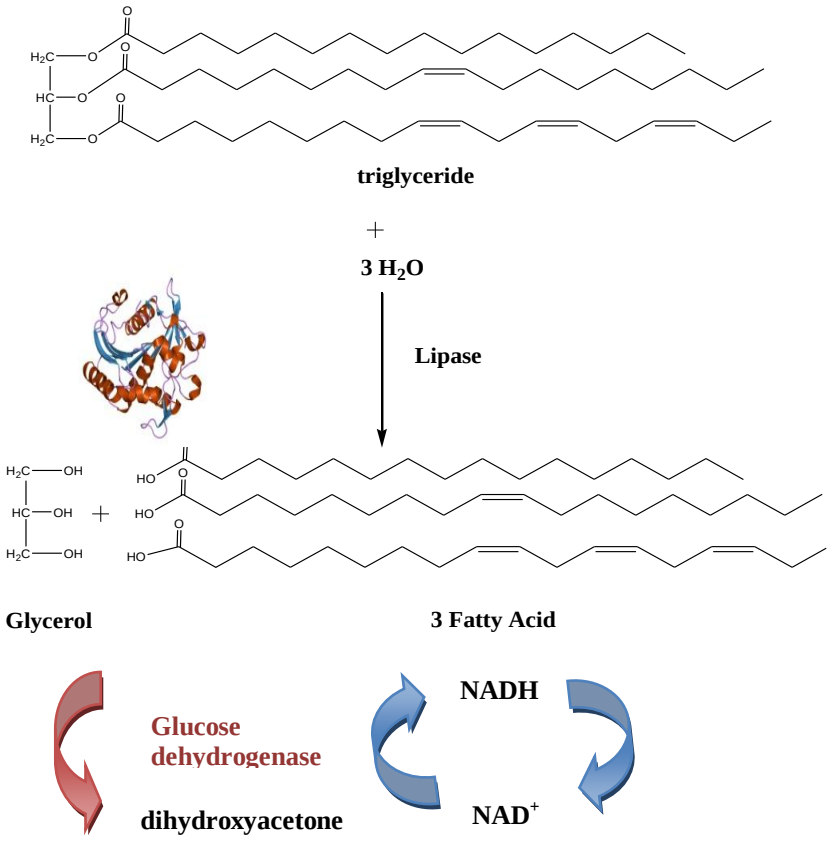

Figure 1. Schematic diagram of biosensor dual enzymatic reaction

Triglyceride (triacylglycerol, TAG or triacylglyceride) is an ester derived from glycerol and three fatty acids; palmitic acid, oleic acid, and alpha-linolenic acid [16]. It is the main constituent of vegetable oil and animal fats. Triglyceride lipase (EC 3.1.1.3) is lipolytic enzyme that hydrolyses ester linkages of triglycerides in water-insoluble lipid substrates (triglycerides), to form fatty acids and glycerol.

It catalyzes the chemical reaction which is shown in Fig. 1. Triglyceride analysis is carried out based on triglyceride hydrolysis and released glycerol detection by glycerol dehydrogenase. The glycerol can then be directly oxidized by glycerol dehydrogenase with the participation of nicotinamide adenine dinucleotide (NAD^+) as the electron acceptor, resulting in the formation of dihydroxyacetone (NADH) and hydrogen ions.

The enzymatically produced NADH can be monitored by an electrochemical sensor through its re-oxidation cycle to NAD^+. Therefore, the triglyceride concentration can then be determined using this biosensor by measuring the output current changes from the enzymatic oxidation reaction producing NADH.

NADH can be directly oxidized at a conventional electrode, but due to its slow charge transfer kinetics, a large over-potential is required. To improve the electron transfer between NADH and the electrode surface, it is require the electrode surface is modified. Nowadays, electrochemical enzyme biosensors are modified by nano-particles which offer high sensitivity; long term stability and low cost detection in biological important molecule determinations [10-15].

Multi wall carbon nanotubes (MWCNTs) can be used as suitable intermediates between electrodes and enzymes, because of their high surface area, high surface to volume ratio, good electrical conductivity and significant mechanical strength [17-19]. Cerium oxide nanoparticles also provide an increased electroactive surface area for loading enzyme and enhancing electron transfer.

In this new triglyceride biosensor, lipase and glycerol dehydrogenase was co-immobilized onto the cerium oxide nanoparticles (CeO_2NPs) and multiwall cabon nanotubes (MWCNTs) on a glassy carbon (GC) electrode which was then fixed by nafion. Nafion, due to its easy fabrication, good electrical conductivity, high chemical stability and good biocompatibility has been widely used as a protective coating material for enzyme immobilization. For improving the detection method, some important parameters were optimized.

2. MATERIALS AND METHODS

2.1. Reagents

Lipase (EC 3.1.1.3 from *Candila rugosa,* CLEA) with specific activity of 2.1 U/mg, glycerol dehydrogenase (EC 1.1.1.6) from *Cellulomonas,* nicotinamide adenine dinucleotide hydrate (NAD) from yeast and triglyceride has been obtained from Sigma–Aldrich. All other chemicals used are of analytical grade. Lipase (1 mg/ml) and glycerol dehydrogenase (1 mg/mL) are freshly prepared in phosphate buffer (10 mM, pH=6.4) prior to being used.

2.2. Biosensor construction

For the construction of triglyceride biosensor, 1 μL suspension solution of 15 mg/mL CeO_2 NPs was deposited onto GC electrode surface (3.0 mm^2) dried at room temperature for 15 min to form a thin NPs-layer. After this procedure, 5 μL solution of lipase, glycerol dehydrogenase and MWCNTs (5 mg/mL) (in phosphate buffer solution (PBS) pH=6.4 and 0.9% NaCl) was coated on the CeO_2 NPs, and dried at room temperature for 10 min to form a biocatalytical enzyme layer. The electrode is kept overnight for enzymes immobilization and then washed with buffer solution. After drying the modified enzymes-MWNT/CeO_2-GC electrode (see Fig. 2), a 10.0 μL nafion solution was dropped onto the electrode and dried for 12 h at 4.0 ^oC to form a film on the modified electrode. When, not in use, the modified GC electrodes were stored in PB solution at 4.0 ^oC.

2.3. Instrumentation

The electrochemical system used for cyclic voltammetric measurements, was a homemade potentiostat connected to a PC PIV outfitted with an analog to digital (A/D) data acquisition board (PCL-818H, Advantech Co.). The A/D board was used to generate an analog waveform and acquire current readings. In the measurements, the memory and CPU requirements of the computer were dictated by the condition of the data acquisition requirements electrochemical software was developed using Delphi 6.0. The potential waveform was repeatedly applied to the working electrode and then the data was acquired, and stored by the software. Also, in this electrochemical setup, the data could be processed and plotted in real time, or the stored data could be loaded and reanalyzed to generate voltammogram.

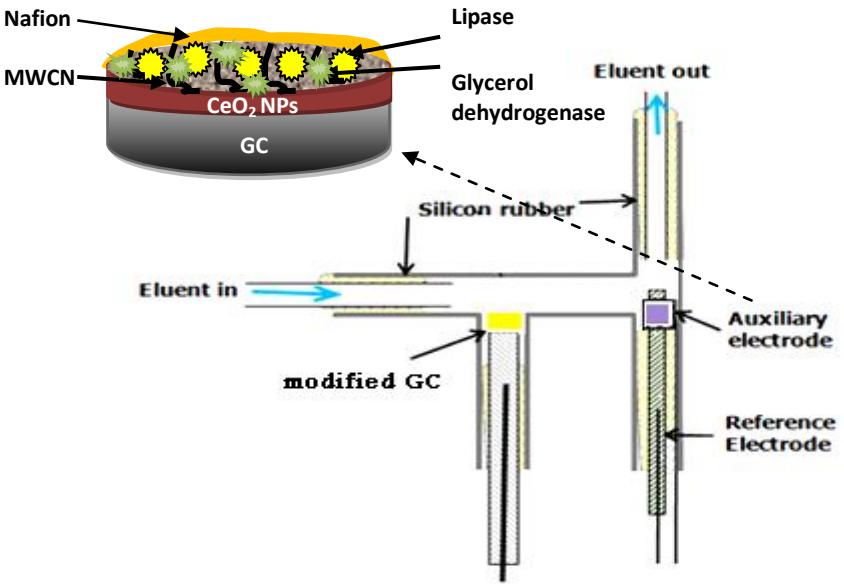

Figure 2. The diagram of the proposed triglyceride biosensor and the electrochemical cell used in flow injection analysis

2.4. Flow Injection Setup

The flow injection analysis equipment integrated with an eight roller peristaltic pump (UltrateckLabs Co., Iran) and a four way injection valve (Supelco Rheodyne Model 5020) with 200 μL sample injection loop [20,21]. The analyte solutions containing NAD^+ in phosphate buffer were introduced into the sample loop by means of a plastic syringe. The electrochemical cell used in flow injection analysis is shown in Fig. 2.

3. RESULTS AND DISCUSSION

It is well known that voltammetric measurement, the sensitivity of the determination is influenced by physical morphology of surface of the biosensor. Consequently in order to investigate the surface, scanning electrode microscopy (SEM) of the biosensor was carried out. Fig. 3 illustrates the typical SEM images of surface of enzymes-MWCNTs/CeO_2 NPs GC.

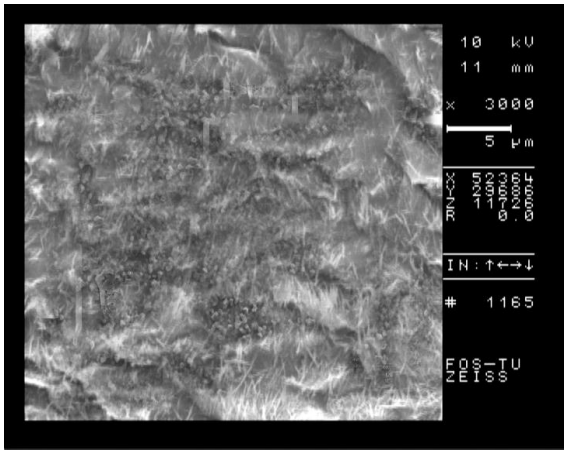

Figure 3. SEM image of the biosensor enzymes-MWCNTs/CeO_2 NPs GC

In Fig. 4, FFT cyclic voltammograms and the changes in the voltammetric response due to injection of the analyte for the enzymes-MWCNTs/CeO_2 NPs GC biosensor in 0.01 M PBS buffer at pH=6.4 is shown. The potential axis on this graph represents potential applied to the biosensor during each sweep, which was -300 to 800 mV at potential sweep rate of 5 Vs^{-1}.

The time axis represents the time passing between the beginning of the flow injection experiment and the beginning of a particular sweep (i.e. it represents a quantity proportional to the sweep number) [22-28]. Also, the graph demonstrates that before injection (in absent of triglyceride) there is no significant peak current in the voltammograms, but by injection of 200 μL of 10 mg/L triglyceride in 0.01 M PBS buffer at pH=6.4 a signal appears at potential of 500 mV.

In this measurement method, the increase in the current of the electrode (at potential of 500 mV) can be due to the generation of H_2O_2 during the oxidation of triglyceride at biosensor surface by

the enzyme. On the other hand, the attachment of the Lipase to high surface area of MWCNTs/CeO_2 nanoparticles facilitates a higher rate of direct electron transfer between the active sites of immobilized enzymes. This can be the reason of existence of the peak current at FFT cyclic voltammograms in the sample was injected.

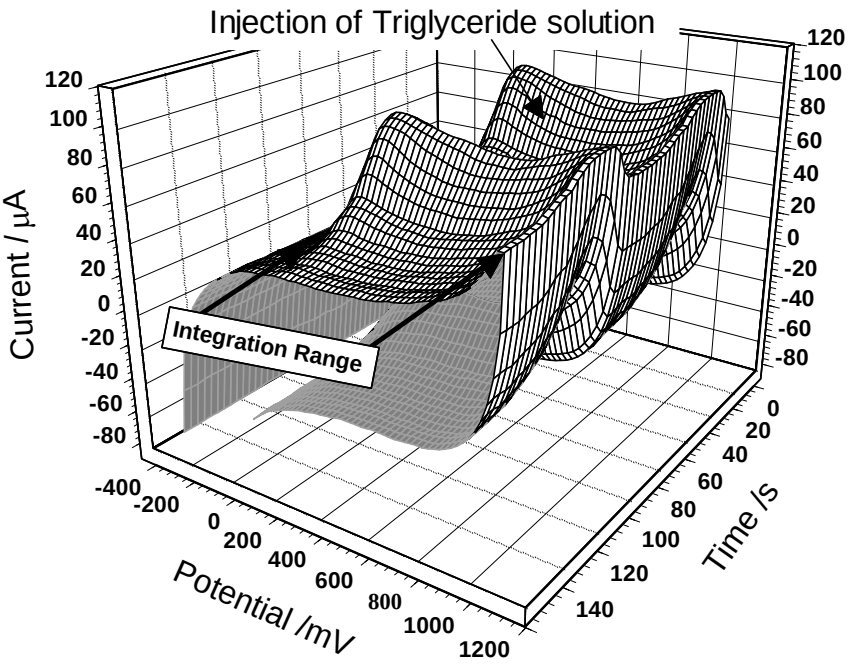

Figure 4. FFTCCV voltammograms of the enzymes-MWCNTs/CeO_2NPs GC electrode without and with injection of 200 μL of 10 mg/L triglyceride in 0.01 M PBS at pH=6.4 in the potential range of -400 to 1100 mV at 5 V/s and the potential Integration range for the current.

The obtained results from injection of standard solutions show that the current increases proportionally by the concentration of triglyceride in the injected. This confirms a good electrocatalytic effect of nanoparticles on fast electron exchange behavior of modified biosensor.

Once FFTCCV is used to monitor a flowing system, triglyceride electrochemical processes will cause a measurable change in the peak current at the voltammograms. According to Fig. 1, triglyceride lipase hydrolyses ester linkages of triglyceride to form fatty acids and glycerol. The released glycerol is then directly oxidized by glycerol dehydrogenase with the participation of NAD^+ in buffer solution, resulting in formation of NADH and hydrogen ions. The enzymatically produced NADH can be monitored by an electrochemical re-oxidation cycle to NAD^+. Therefore, the triglyceride concentration can then be determined using this biosensor by measuring the output current changes from the enzymatic oxidation reaction producing NADH. In this detection method the current passing through the electrode was sampled only during the potential ramp. Another statistical method for improving the S/N ratio is the signal integration method [28]. Based on this method the electrode response was calculated by integrating the current in a selected potential range (E_1 to E2, see Fig.4),

$$Q_t = \int_{E1}^{E2} \Delta i(t, E) dE \quad (1)$$

where Q_t is the charge under the curve in the range E_1 to E_2. In fact, the signal integration operation not only minimizes the noise resulting from fluctuation in the reference electrode potential, but also, eliminates or reduces the contribution of the noise frequencies in the background current.

The integration of net current changes is applied over the selected scanned potential range. In this method, *ΔQ* is calculated based on the all-current changes at the CV. A total absolute difference function (*ΔQ*) can be calculated by using the following equation:

$$\Delta Q\ (s\tau) = \Delta t \left[\sum_{E=E_i}^{E=E_f} \left| i(s, E) - i(s_r, E) \right| \right] \quad (2)$$

Where, *s* is the sweep number, τ is the time period between subsequent potential scan, *Δt* is the time difference between two subsequent points on the cyclic voltammograms, *i* (*s*, *E*) represents the current of the cyclic voltammograms recorded during the s-th scan and $i(s_r, E)$ is the reference current of the FFTCCV voltammograms. E_i and E_f are the initial and the final potential, respectively, for integrating of current. This integration range for the current is shown in Fig 4. The reference cyclic voltammogram was obtained by averaging a 3 to 5 cyclic voltammograms, recorded at the beginning of the experiment before injection of the analyte.

3.1. Optimizing the experimental parameters

In this detection method, sensitivity of the measurement can be influenced by potential scan rate. This is typically due to kinetic factors of the reactions on the surface of the biosensor, and also the instrumental limitations. From this point of view, examination the change in sensitivity of the technique by the sweep rate is necessary.

With the purpose of study the influence of FFTCCV scan rates and the elunet flow rate on the sensitivity of the detector response, at different scan rates (from 0.5 to 10 V/s) and the eluent flow, solutions having a concentration of 50 mg/L of triglyceride were injected, and the responses of the detector were recorded. The obtained results are presented in Fig. 5.

As it is shown the Fig. 5, the detector exhibits the maximum sensitivity at 5 V/s of scan rate and 4 mL/min of the flow rate. The effects of the sweep rate on the detection performance can be taken into consideration from three different aspects: First, speed in data acquisition, second, kinetic factors of electrochemical processes, finally, the flow rate of the eluent which controls the time retention of the solution zone in the detector. The effect of flow rate on the S/N ratio can be explained under two topics. First, the effect of the flow rate on the zone broadening of the injected sample; second, the relationship between data acquisition (or voltammetry sweep rate) and flow rate in a flowing solution.

Two main factors may contribute to the zone broadening are diffusion and convection. Migration of the analyte from the zone to eluent, due to diffusion or convention, cause a decline in sample concentration, consequently, the electrode response would lower than actual value. The zone broadening effect can be electrode response can be evaluated based on dispersion coefficient, D_{max},

$$D_{max} = Co/C_{max} = R_{max}/R \quad (3)$$

Where C_{max} and R_{max} are the sample concentration near the electrode and the corresponding response respectively. C_o and R are the actual concentration of sample in the zone and the corresponding response. Dispersion factor, in FIA can be minimized by optimization flow injection loop (see Fig. 5). Unlike the chromatographic methods, in FIA zone broadening is not a serious problem, it can affect only the detection limit of the measurements. The main reason for application of high scan rates can have a negative effect on electrochemical processes during the potential scanning.

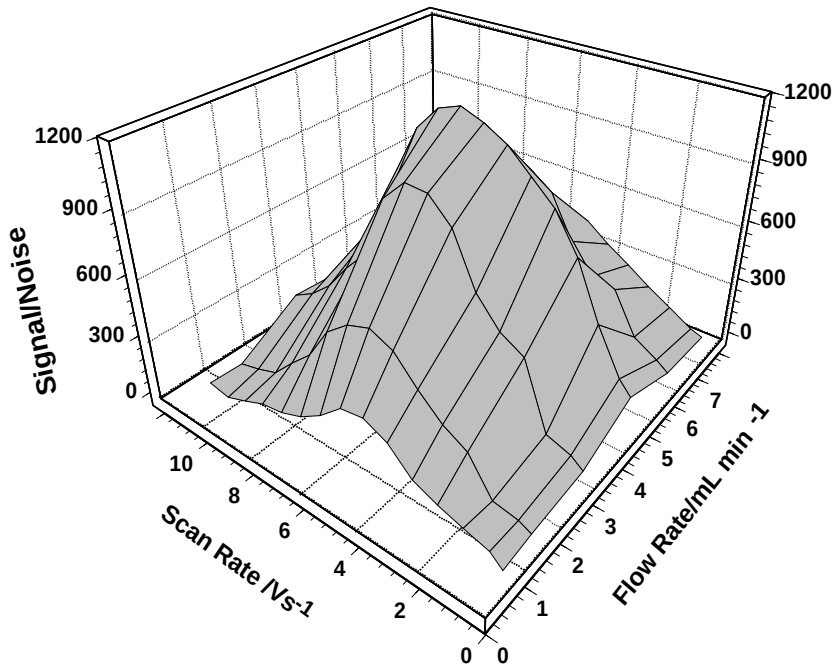

Figure 5. Effect of the sweep rate and effect of flow rate on the response of the enzymes-MWNT/CeO_2NPs GC electrode to injections of 50 mg/L triglyceride

3.2. Effect of pH on the sensitivity of the biosensor

Fig. 6 shows the effect of the solution pH and Lipase concentration on response of the biosensor. It is proven that the pH value of the solution has a significance influence on the performance of the biosensor. This mainly is because of the activity of the immobilized Lipase is pH dependent. The results show that the response of the biosensor increases in the pH range of 5.0–6.0, the current response decreases and the background current increased at higher pH values. In fact, the

best operating valve for biosensor is at pH 6.4. In addition, the graph shows that the minimum concentration of enzyme in the solution for making the biosensor is 0.9 mg/mL.

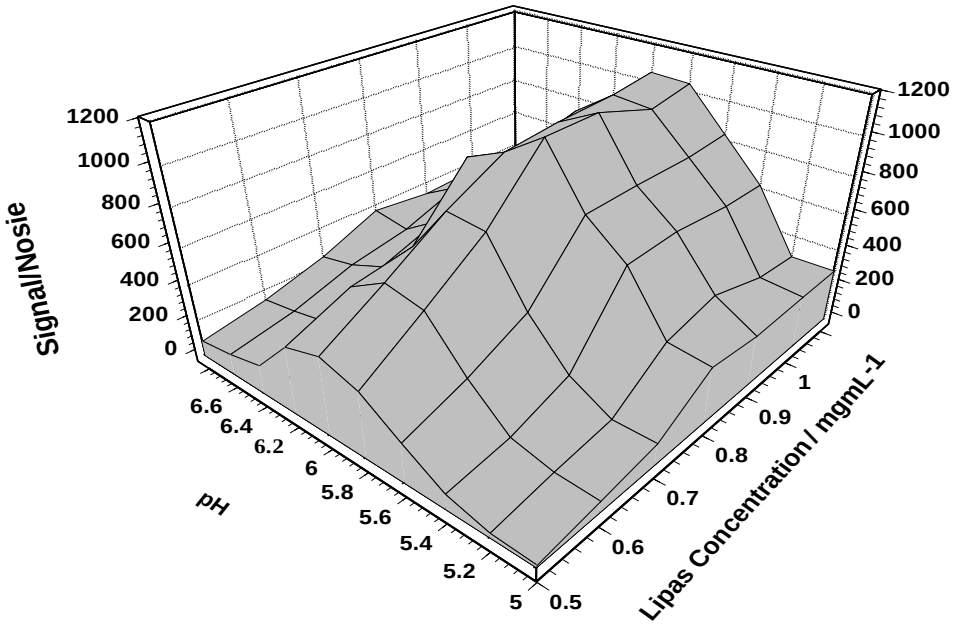

Figure 6. The effect of pH and Lipase concentration on response of the enzymes-MWCNTs/CeO_2 NPs-modified GC electrode to injections of 50 mg/L triglyceride

3.3. Calibration curve and biosensor characterization

From an analytical point of view the relation between concentration of analyte and the electrode response, called the calibration curve, is the most important. It is expected that for most substances electrode response is proportional to electrode coverage. This is obviously the case when ΔQ due to interaction of triglyceride is monitored. Fig. 7 shows response and reproducibility of the biosensor, to three injections of standard solutions of triglyceride.

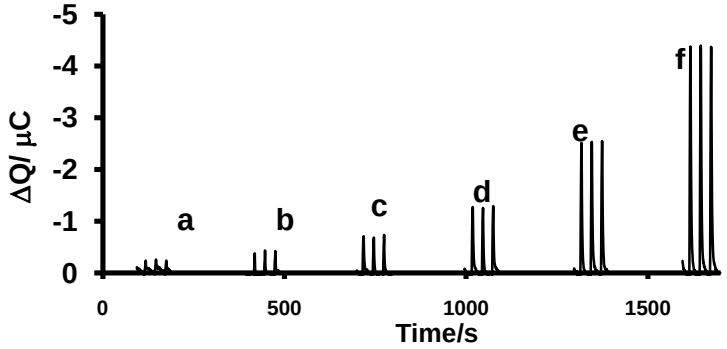

Figure 7. Response of the enzymes-MWCNTs/CeO_2 NPs modified GC electrode to triglyceride in upon the following concentrations: a, 1; b, 2; c, 4; d, 8; e, 15; f, 25 mg/L.

Results shown in this figure represent the integrated signal for 3 consecutive flow injections of the standard solution. Fig. 8 illustrates a typical ΔQ response of the modified electrode on a standard solution of triglyceride (from 1.0 to 100.0 mg/L in 0.01 M PBS solution, pH=6.4). The experimental conditions were set at optimum values in order to obtain the best detection limits. Under optimized conditions, the steady-state FFTCCV showed a linear dynamic range of 1 to 100 mg/L (Fig. 8). A correlation coefficient of R=0.997 with %R.S.D. values ranging from 0.34–4.1% across the concentration range studied were obtained following linear regression analysis.

As mentioned above the electrode response could be expressed in various ways as peak heights or peak areas. For this reason, the magnitude of the flow-injection peaks depends on the choice of the data processing methods. Measurements carried out for small analyte concentrations allow the estimation of the detection limit C_{DL}:

$$C_{DL} = \frac{3s_b}{slope}, \tag{4}$$

Where s_b is the standard deviation (or noise) of the baseline around the flow-injection peak. The linearity was evaluated by linear regression analysis, which calculated by the least square regression method. The detection limit, estimated based on signal to noise ratio (S/N=3), was found to be 0.5±0.01 mg/L.

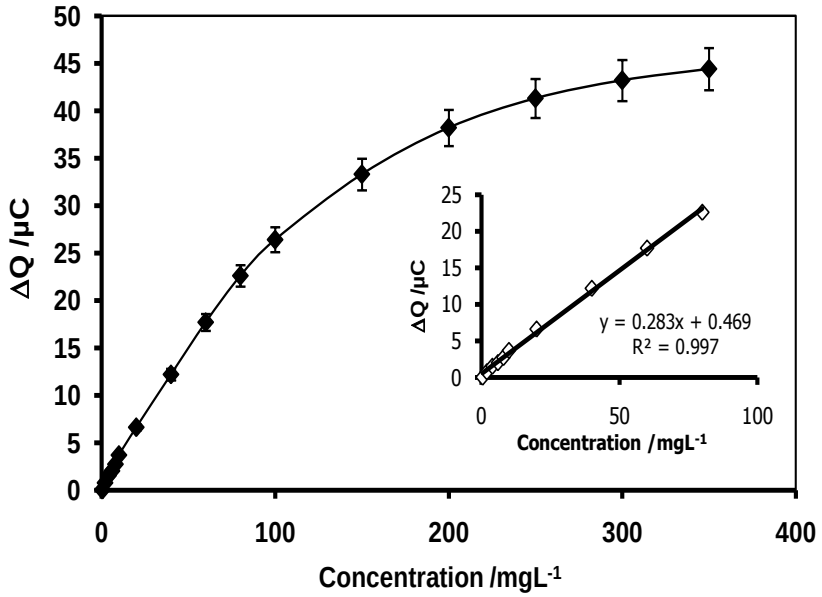

Figure 8. The calibration carve for the enzymes-MWCNTs/CeO_2 NPs GC electrode by injection of 200 μL of standard solution of triglyceride in 0.01 M PBS at pH=6.4 in the potential range of -400 to 1100 mV at 5 V/s.

4. CONCLUSIONS

A highly sensitive triglyceride biosensor has been fabricated by modifying the GC electrode surface with MWCNTs/CeO_2 NPs. A high producible response time less than 25 s and detection limit of 0.5 mg/L was observed from the fabricated biosensor. To the best of our knowledge, this is the first presentation of a very accurate with a low detection limit method which is used for triglyceride biosensors based on MWCNTs/CeO_2 NPs modified electrodes.

The results of a long-term (more that 55 days) storage stability of the biosensor showed that the sensitivity retained 95.2% of initial sensitivity and after that time gradually decreases afterwards might be due to the loss of the catalytic activity.

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